BRIEF REPORT

The Impact of Freeze-Thaw Cycles on Epinephrine

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Objectives.—Epinephrine is the first-line medical treatment for anaphylaxis, a life-threatening allergic syndrome. To treat anaphylaxis, backcountry recreationalists and guides commonly carry epinephrine autoinjectors. Epinephrine may be exposed to cold temperatures and freezing during expeditions. An epinephrine solution must contain 90% to 115% of the labeled epinephrine amount to meet United States Pharmacopeia standards. The purpose of this study was to determine whether freeze-thaw cycles alter epinephrine concentrations in autoinjectors labeled to contain 1.0 mg/mL epinephrine. A further objective was to determine whether samples continued to meet United States Pharmacopeia concentration standards after freeze-thaw cycles.

Methods.—Epinephrine from 6 autoinjectors was extracted and divided into experimental and control samples. The experimental samples underwent 7 consecutive 12-hour freeze cycles followed by 7 12-hour thaw cycles. The control samples remained at an average temperature of 23.1°C for the duration of the study. After the seventh thaw cycle, epinephrine concentrations were measured using a high-performance liquid chromatography assay with mass spectrometry detection.

Results.—The mean epinephrine concentration of the freeze-thaw samples demonstrated a statistically significant increase compared with the control samples: 1.07 mg/mL (SD $\pm$ 8.78; 95% CI, 1.04 to 1.11) versus 0.96 mg/mL (SD $\pm$ 6.81; 95% CI, 0.94 to 0.99), respectively. The maximal mean epinephrine concentration in the experimental freeze-thaw group was 1.12 mg/mL, which still fell within the range of United States Pharmacopeia standards for injectables (0.90 to 1.15 mg/mL).

Conclusions.—Although every attempt should be made to prevent freezing of autoinjectors, this preliminary study demonstrates that epinephrine concentrations remain within 90% to 115% of 1.0 mg/mL after multiple freeze-thaw cycles.

Key words: anaphylaxis, epinephrine, adrenaline, autoinjector, drug stability

Introduction

Anaphylaxis is a life-threatening allergic syndrome that may be triggered by an insect sting, contact with certain plants or other substances, medications, and certain foods. It is estimated that 1% to 2% of the general population is at risk for anaphylaxis, and that the global prevalence of those with epinephrine prescriptions may be as high as 2%.¹ Epinephrine is the first-line medical treatment for anaphylaxis, and administering epinephrine

can rapidly reverse bronchospasm and other life-threatening manifestations of the reaction.

Backcountry recreationalists and guides commonly carry epinephrine in the form of an epinephrine autoinjector. The adult dose autoinjector is a single-use, self-administered mechanical device that delivers 0.3 mL of 1:1000 concentration epinephrine (0.3 mg) intramuscularly via a spring-loaded needle and syringe. In the United States and Canada, one such autoinjector (Figure 1) is marketed under the trade name EpiPen.² Those who are known to be severely allergic to specific triggers or have experienced anaphylaxis in the past should carry autoinjectors, or another form of epinephrine, with them in the wilderness. In addition, wilderness guides and instructors should consider carrying epinephrine in their medical kits to address unexpected anaphylactic reactions in the backcountry.³

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Figure 1. EpiPen autoinjector that has been disassembled to show internal ampule. Solution has been removed.

In backcountry environments, epinephrine formulations may be exposed to extreme temperatures. The manufacturer of EpiPen provides prescribing information for the labeled recommended storage, which is protection from ultraviolet light and storage between 20°C and 25°C with excursions permitted to between 15°C and 30°C.² The manufacturer recommends replacing the product if it has been accidentally refrigerated. This may not be possible on an extended backcountry expedition.

It is well established that epinephrine solutions are unstable when exposed to high temperatures (ie, in excess of 25°C) or exposure to direct ultraviolet light.⁴ Epinephrine also has a limited shelf life, with significant degradation occurring after expiration of drug samples.⁵ However, it has been reported in previous literature that epinephrine solutions within expiration dates are

stable in refrigeration and at cooler temperatures, and refrigeration even has been described as a technique to prolong shelf life of epinephrine solutions,⁴ despite current manufacturer recommendations. For example, when epinephrine was cooled to 2.5°C and subsequently returned to room temperature (23°C), there was no statistically significant change in concentration of epinephrine.⁶ In another study, Zenoni et al⁷ stored epinephrine solutions in Luer-lock syringes at temperatures between 2°C and 8°C for up to 24 weeks and detected no degradation of epinephrine. In the current literature, there are no studies addressing epinephrine stability after a single freeze or after multiple freeze-thaw cycles.

The purpose of this study is to determine whether there is a change in epinephrine concentration after samples are exposed to freezing temperatures followed by thawing. These results will help guide the use of epinephrine autoinjectors while on backcountry expeditions in cold environments where freezing may be unavoidable. Based on previous literature and knowledge that epinephrine degradation reactions of oxidation and sulfonation happen more readily when the compound is exposed to heat and light, we hypothesized that epinephrine concentration would not be affected by freezing temperatures (Figure 2).

Methods

PREPARATION AND STORAGE OF EPINEPHRINE EXPERIMENTAL AND CONTROL SAMPLES

High-performance liquid chromatography (HPLC) has been used in previous studies of epinephrine stability to determine concentration values in drug solutions.^{4,5} Our

Figure 2. Degradation reactions of L-epinephrine. Adrenochrome creates a colored precipitate within epinephrine solutions. Discoloration of EpiPens indicates degradation has occurred and the autoinjector should be discarded.

assay used HPLC followed by mass spectrometry to ensure that epinephrine was identified by highly accurate mass-to-charge ratios and reliably quantified. Epinephrine was removed from the internal glass ampules of 6 autoinjectors (Mylan Specialty, Basking Ridge, NJ; lot number 3GM581; expiration May 2015, experiment performed April 2014). The commercially formulated drug solution from each autoinjector was divided into 2 screw-topped amber glass vials (Aligent Technologies, Santa Cruz, CA) and designated as samples for either control or experimental groups. The experimental samples underwent a 7-day consecutive freeze-thaw cycle: the samples were placed in a -25°C freezer from 1900 hours to 0700 hours (average temperature of -25.4°C), then allowed to thaw at room temperature from 0700 hours to 1900 hours (average temperature of 22.7°C). The control samples remained in sealed glass vials at room temperature (average temperature of 23.1°C), away from direct sunlight, for the 7-day duration of the study. Temperatures were recorded and compared among 3 temperature data loggers (Lascar Electronics, Whiteparish, UK) throughout the freezing and thawing cycles.

After the seventh thaw cycle, all vials were divided into 5 light-protected test tubes. All underwent individual 10 000-fold serial dilutions with 0.1% formic acid. Aliquots of each (50 μL) then were loaded promptly into separate autosampler vials. Calibration standards in 0.1% formic acid and quality controls in 0.1% formic acid were interspersed systematically in autosampler vials and used to verify HPLC-mass spectrometry instrument accuracy and reliability. Epinephrine- d_3 (Santa Cruz Biotechnology, Dallas, TX) was used as an internal standard for this assay (50 μL of 100 ng/mL solutions in 0.1% formic acid). Quality control samples consisted of epinephrine (Aldrich, Milwaukee, WI) aliquots at known concentrations of 40, 100, and 170 ng/mL. Epinephrine calibration standards consisted of epinephrine (Sigma, St. Louis, MO) at concentrations from 20 to 200 ng/mL in 0.1% formic acid for a total of 15 data points used for HPLC-mass spectrometry peak integration calibration for drug quantification. Along with study samples, the analytical run included calibration standards, internal standards of epinephrine- d_3 , and quality control samples, which all were analyzed during a single HPLC run using the same instrument.

LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY ANALYSIS

An Agilent 1100 liquid chromatograph (Agilent Technologies, Santa Clara, CA) interfaced with a Thermo-Finnegan (San Jose, CA) TSQ 7000 triple quadrupole mass spectrometer (MS-MS) was used for the analysis.

The system was operated by an Xcalibur data systems. A Zorbax Eclipse XDB-C18 4.6×150 mm (Agilent) liquid chromatography column at 35°C was used. The mobile phase was 10 mM ammonium acetate pH 5 in methanol (50:50) at a flow rate of 0.2 mL/min.

During the MS-MS analysis, positive ion electrospray was used for ionization. The capillary temperature was 250°C . Selected reaction monitoring was used for the analysis. The following m/z transitions were monitored: epinephrine, $184 \rightarrow 166$; and epinephrine- d_3 , $187 \rightarrow 169$. A calibration curve generated from analysis of the calibration standards was used to extrapolate the epinephrine concentration in the analytical quality controls and the dilutions of the study samples, for a total of 30 control concentration values and 30 experimental (freeze-thaw) concentration values. Values obtained were then compared with the labeled amount of epinephrine in EpiPens (1.0 mg/mL) for both controls and experimental samples. Significant loss or gain of epinephrine was defined as failure to meet an epinephrine concentration of between 90% and 115% of the labeled amount (0.9–1.15 mg/mL), as United States Pharmacopeia (USP) standards dictate.⁸ This assay quantified only the parent drug at its known m/z peak location and did not analyze known degradation products of epinephrine.

STATISTICS

Means, SDs, and 95% CIs for the concentration values for the control and experimental groups were calculated for the overall study and stratified by which EpiPen was the source of the sample. Differences among concentration values for the related sets of experimental samples and related sets of control samples were analyzed by random effects analysis of variance. The random effects approach was used to account for relatedness within the 10 aliquots from each autoinjector, including 5 of each autoinjector in the control and 5 in the experimental arm of the study, while also accounting for differences between the aliquots from different pens. Statistical significance between experimental and control groups was defined as $P \leq .05$. All analyses used SPSS software v.22.0 (IBM SPSS Inc, Chicago, IL).

Results

Averages and SDs of the measured epinephrine concentration values from each EpiPen aliquot (control and experimental) are shown in the [Table](#).

Random effects analysis of variance and t test analysis of all control versus experimental concentration values yielded a P value of $< .001$. The SD of all values was 9.53. Control samples had a 95% CI of 0.939 to 0.990

Table. Measured epinephrine concentration values for both control and experimental EpiPen aliquots after freeze-thaw cycling^a

EpiPen sample	Control (mg/mL)	Experimental (mg/mL)
A	0.932 ± 2.30	1.006 ± 7.35
B	0.954 ± 2.62	1.045 ± 12.57
C	0.946 ± 3.19	1.097 ± 2.50
D	1.010 ± 3.05	1.122 ± 3.42
E	0.996 ± 14.84	1.116 ± 13.28
F	0.951 ± 4.82	1.058 ± 4.88
All EpiPens	0.965 ± 6.81	1.078 ± 8.78

^a Values reported as average ± SD.

mg/mL. Experimental samples had a 95% CI of 1.041 to 1.106 mg/mL.

Discussion

Multiple freeze-thaw cycles did not result in a detectable significant loss (concentration <0.90 mg/mL) of epinephrine in our study. The mean concentration values of all measured control and experimental samples fell within 0.90 mg/mL to 1.15 mg/mL, and thus continued to meet USP monograph standards for epinephrine injections.

Loss of epinephrine concentration, if indeed occurring, would suggest that epinephrine has undergone one of several known degradation pathways well-characterized in the literature.⁴ These involve reactions with stabilizing salts (sulfonation) and oxidation reactions to form adrenochrome, as well as racemization of therapeutically active L-epinephrine to less-active D-epinephrine (Figure 2). Adrenochrome results in discoloration of epinephrine solutions, and we observed no visible discoloration of our samples after freeze-thaw cycles that would suggest oxidation to adrenochrome had occurred. The stabilizing salts present in 0.3 mL of EpiPen solution are 1.8 mg sodium chloride and 0.5 mg sodium metabisulfite, dissolved in water with hydrochloric acid to adjust the solution pH to a range of 2.2 to 5.0.² This stabilizing solution should not undergo any significant reaction under freezing conditions, and the stabilizing salts of the solution are present to prevent oxidation of epinephrine. Other than racemization of epinephrine, all described degradation pathways significantly change the molecular weight of epinephrine and thus would affect the concentration of the drug molecule detected with MS-MS analysis. Our results suggest that such degradation did not occur.

Curiously, we observed a statistically significant increase in the amount of epinephrine after multiple

freeze-thaw cycles. A clear explanation for this increase in epinephrine concentration is not apparent. One possibility is that the concentration of the epinephrine content increases through the loss of solution during the freezing and thawing of the samples, despite the use of sealed vials used to store both control and experimental solutions. Samples were not weighed after freeze-thaw cycles, and this should be performed with any follow-up studies involving freeze-thaw cycles. There is also possibility of analytical error or preparation error in our assay. Every attempt was made to prevent analytical error, including a single-run HPLC MS-MS assay of all samples quantified by the same instrument. Analysis via HPLC MS-MS followed sample and internal standard dilutions and loading of samples into autosampler vials. Epinephrine solutions for both control and experimental samples were also derived from the same EpiPens with the same lot number, in an attempt to minimize any discrepancies in drug concentration that may have occurred during manufacturing. Although obviously a research standard in pharmaceutical chemistry, HPLC MS-MS assays are still fallible and require techniques to enhance method validation. The well-described matrix effect, which involves alteration of ionization efficiency of the instrument by the presence of coeluting substances, may lead to error in both accuracy and sensitivity of quantification.

Future epinephrine delivery systems may address the challenges of carrying autoinjectors in extreme environments. Recently, sublingual forms of epinephrine have been suggested as a possible method of epinephrine administration by laypersons, a method that could help solve the problem of freezing solutions.¹⁰ Another question is whether the EpiPen drug delivery mechanism would be more prone to failure after repeated freezing and thawing. To compare concentrations of epinephrine derived from the same EpiPen, our study removed epinephrine solution from its original autoinjector packaging (Figure 1) and subdivided it. In future studies, intact EpiPens subjected to extreme temperatures should be tested for mechanical injection failure and failure of the glass storage ampule. Before beginning this study, we froze an intact EpiPen and later discharged the thawed autoinjector with no defects noted in the injector or in the glass ampule that was later removed. This should be repeated with multiple samples.

Lieberman et al¹ state that treatment of anaphylaxis requires aqueous epinephrine in a 1:1000 dilution (1 mg/mL), 0.2 to 0.5 mL injected intramuscularly or subcutaneously every 5 minutes as necessary to control symptoms. From the results of this small study, it appears that epinephrine that has been frozen and

thawed still has the required parent drug concentration to remain efficacious for use in anaphylactic emergencies.

LIMITATIONS

Epinephrine is a chiral molecule, with several-fold greater bioactivity of L-epinephrine over D-epinephrine.⁹ Therapeutic solutions of epinephrine contain L-epinephrine, and racemization reactions to D-epinephrine in drug formulations are minimized by maintaining a low pH in solutions.⁴ L-Epinephrine should predominate in epinephrine injectables that are obtained before the expiration date. A criticism of the USP standard for epinephrine is that the assay does not list a specific enantiomeric purity or concentration of injection solutions, and thus does not directly take into account the concentrations of L-epinephrine vs D-epinephrine.⁵ Our assay was similar to that of the USP monograph for epinephrine injections in that we focused on measuring concentration of epinephrine and did not subject our samples to optical polarimetry. Thus a major limitation of our study is disregard of the racemization pathway of degradation of the molecule. We measured concentration changes in epinephrine without quantifying L-epinephrine vs D-epinephrine contents, reasoning that drugs meeting USP concentration standards should be suitable for administration to patients. However, the possibility of drug racemization cannot be ignored, and future studies should address this concern. Several methods, such as optical polarimetry and chiral HPLC, are available for chiral qualification of epinephrine solutions. These methods were beyond the scope of this project.

Another limitation of this study is the relative changes made in the storage of all samples from the EpiPens. To replicate the most likely expedition scenario, intact autoinjectors would need to be frozen and thawed repeatedly and analyzed for any change in concentration of active drug. As stated above, we thought that subdividing control and experimental samples from the same autoinjector would control for any possible differences in concentration and prevent losses of solution on disassembly, which could have resulted in a smaller sample size if solution losses were large. Because ampules of EpiPen autoinjectors are glass, we did not believe storage in amber glass vials would represent a significant difference in storage of solution.

Finally, this study analyzed concentration values from 6 EpiPens. Concentration value analysis could thus be influenced by the small *n* value, as well as the inherent variability of the HPLC instrument, discrepancies in serial dilutions, and potential contamination of drug samples.

Conclusions

After repeated freezing and thawing, the epinephrine concentrations in commercial, marketed, and United States Food and Drug Administration–approved formulations measured by our assay remained within USP manufacturer standards of 90% to 115% of the original labeled concentration of epinephrine, but after freeze-thaw cycles, epinephrine concentrations showed a statistically significant increase that remains unexplained by our study. Thus, epinephrine concentrations still measure within therapeutic ranges despite a 7-day exposure to extreme conditions meant to simulate the potential environmental conditions of a weeklong backcountry trip or expedition. Freezing of epinephrine should be prevented in extreme environments because frozen medication cannot be administered in an emergency. Also, published manufacturer standards for storing medications should be followed whenever possible.

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